

Angiotensin-Converting Enzyme: II. Pulmonary Endothelial Cells in Culture

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Direct studies of the function of a given cell type often require that the cell type be obtained in pure culture. A number of different specific metabolic activities have been attributed to pulmonary endothelial cells, yet with few exceptions the conclusions were based on indirect evidence. Thus, to improve our ability to examine directly for specific metabolic activities, we began a program to obtain pulmonary endothelial cells in culture. Two methods have been developed: (1) cells can be obtained from pulmonary artery and vein of large animals (cow, pig), and (2) cells can be obtained from the microvasculature of small animals (rat, guinea pig, and rabbit). The latter technique can also be used to obtain cells from a lobe of lung from large animals and may be adaptable for use with human tissue. In the first technique, pulmonary arteries, free of blood, are filled with collagenase (0.25%, 500 units) in Puck's saline for 25 min. The collagenase mixture containing cells is removed and centrifuged. The pellet is resuspended and seeded into culture flasks. In the second method, lungs are perfused (artery to vein) with Krebs-Henseleit solution until the effluent is blood-free. Collagenase (0.25%, 500 units) is introduced, and the lungs are then perfused in the opposite direction (vein to artery) until the flow stops spontaneously (ca. 15 min). The detached cells are collected and seeded as before. The endothelial cells attach as small clumps (10-50 cells). Those flasks which contain more than 95% endothelial cells (by phase microscopy) are retained for culture and the lines are purified using differential adherence procedures. The cells grow as monolayers with a cobblestone appearance. They contain Weibel-Palade bodies. They possess converting enzyme activity and are reactive with antibodies to converting enzyme, Factor VIII and α_2 -macroglobulin. The cells synthesize prostaglandins and related substances. In addition, they possess ADPase and synthesize angiotensin-converting enzyme.

Introduction

One of the technical limitations to progress in understanding of specific functions of a particular cell type is the availability of that cell type in pure culture. Over the past 12 years, a number of different specific metabolic activities have been attributed to pulmonary endothelial cells. The cells can selectively process adenine nucleotides and biogenic amines (1, 2). In addition, endothelial cells are capable of forming prostaglandins such as PGE₂ (3-5) and PGI₂ (6, 7). Endothelial cells appear to have a great capacity for inactivating kinins and for converting angiotensin I into angiotensin II (3, 8), and in this sense endothelial cells of the pulmonary vascular bed can regulate the hormonal composition of sys-

temic arterial blood. In addition, endothelial cells appear to possess a number of hemostatic factors including α_2 -macroglobulin (9), plasminogen activator (10) and factor VIII antigen (11).

By and large, metabolic activities attributed to pulmonary endothelium were based on conclusions drawn from indirect evidence. Perhaps the most direct method for examining for unique or characteristic properties of a given line of endothelial cells is to use isolated cells (12-14) or cells in culture. Others have developed methods for isolating and culturing endothelial cells from human umbilical vein (15-18). Similar techniques have been used to isolate and culture endothelial cells from portal vein and aorta (19-23). Therefore to improve our ability to examine directly for specific metabolic activities of pulmonary endothelium, we have begun a program to obtain pulmonary endothelial cells in culture. Two methods have been developed: cells can be obtained from pulmonary artery and vein of large animals

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(cow and pig); alternatively cells can be obtained from the microvasculature of small animals (rats, guinea pig and rabbit), animals used commonly in studies of "nonventilatory functions" of the lungs. The latter technique can also be used to obtain cells from a lobe of lung from large animals and may be adaptable for use with human tissue obtained by open biopsy.

Materials and Methods

Isolation and Culture of Pulmonary Artery Endothelial Cells

Pulmonary arteries from newborn or unweaned calf (veal) are obtained within 30 min of slaughter. Calf hearts with attached great vessels are transported at 4°C to the laboratory where they are rinsed with Puck's saline. Each pulmonary artery is separated from the aorta and trimmed of excess fat and connective tissue. The pulmonary artery is tied as close to the heart as possible and the vessel is removed. Each vessel is washed by immersion in three changes of Puck's saline containing 3x strength antibiotics (Biostat 100, 3 ml/l.). When all of the vessels have been trimmed and washed, they are placed in a beaker of Puck's saline with 3x antibiotics and stored at 4°C for approx. 90 min. This storage step appears to reduce the likelihood of subsequent bacterial contamination. The vessel segments are then warmed to 37°C in a shaking waterbath. Each pulmonary artery segment is drained of excess saline and filled with a solution of 0.25% collagenase (Worthington type CLS II) in Puck's saline containing normal strength antibiotics (Biostat 100, 1 ml/l.). The free end of each artery is then clamped with a large hemostat. The arteries are suspended in a 2 l. beaker of phosphate buffered saline and incubated at 37°C in a shaking waterbath for 25 min. The arteries are then removed and the exteriors blotted dry on sterile tissue. Each artery is then suspended by the ligature over a small beaker or centrifuge tube and the hemostat is released. The collagenase mixture containing cells is decanted into 15ml tubes and centrifuged at 750g for 10 min at 4°C. The supernatant is discarded and the pellets washed by resuspending and repelleting twice with cold medium 199 (without fetal calf serum). The final pellets are then resuspended in 1 ml of medium 199 and seeded into 25 cm² tissue culture flasks containing 4 ml of medium 199 with 20% fetal calf serum and normal strength (1 ml/l.) Biostat 100. The flasks are capped loosely and placed in a CO₂ (5%) incubator at 37°C. The cells show the greatest plating efficiency when seeded as small clumps rather than as monodispersions. Therefore, hemocytometer counts may not be

accurate. However, cells seeded at densities of approx. $1.5-3 \times 10^5$ per flask reach confluency within 5 days. At confluence, the cells reach a density of about $4 \times 10^4/\text{cm}^2$ (10^6 per flask). The medium is changed every two days.

The cells can be subcultured as follows: Flasks are washed for approx. 10 min with 5 ml of Ca²⁺- and Mg²⁺-free Puck's saline at 37°C. The saline wash is aspirated off and the cells are incubated with 0.05% trypsin with 0.02% EDTA in Ca²⁺- and Mg²⁺-free Puck's saline (5 ml at 37°C). The cells are monitored on an inverted microscope to determine the optimal length of time of exposure to the trypsin-EDTA solution. We have found that after 3 min the small, polygonal endothelial cells begin to lift off, while the larger, flatter smooth muscle cells are still adherent to the flask. With a minimum of agitation, the enzyme mixture containing cells is transferred, by using a 5 ml pipet, to 15 ml conical centrifuge tubes. The cells are centrifuged at 750g for 10 min. The trypsin solution is removed by aspiration and the pellet is resuspended in a small quantity of medium 199 without fetal calf serum. The flasks are usually split two for one. Each new flask is seeded with approximately 10^5 cells. The doubling time for bovine pulmonary artery endothelial cells is approximately 24 hr. Flasks which are predominantly endothelial (less than 5% contamination with smooth muscle cells) can be purified by successive subculture; taking advantage of the difference in attachment and detachment rates of the two cell types. Trypsin preferentially detaches bovine endothelial cells; thus, short periods (2 to 3 min) of exposure to trypsin prior to transferring cells tend to leave smooth muscle cells adherent to the flasks. Furthermore, endothelial cells are the first to reattach. Hence, cells are seeded into new flasks and incubated for 1 hr at 37°C. Medium and any free-floating cells are removed by aspiration and replaced with fresh medium containing fetal calf serum and antibiotics.

Isolation and Culture of Endothelial Cells From the Lungs of Small Animals

The techniques described above for the isolation and culture of endothelial cells obtained from bovine pulmonary artery have provided useful information on specific metabolic activities of pulmonary endothelium (3). The techniques are not however suitable for obtaining endothelial cells from the vessels of small animals or from the microvasculature. We have, therefore, developed a technique for obtaining endothelial cells from the lungs by vascular perfusion with collagenase in a HEPES buffered salt solu-

tion. The surgical procedure and techniques for collection of cells are similar using rats, rabbits or guinea pigs of either sex (Fig. 1).

The following describes the perfusion system used with a female New Zealand white rabbit of 2.16 kg. A tracheostomy was performed and a tracheal tube

was inserted, secured with a ligature and attached to a Harvard respirator (tidal volume 18 ml, 45 strokes per minute, inspiration to expiration ratio of 0.45). Heparin (5,000 units) was injected intravenously. Ligatures were placed around the pulmonary artery and around the left atrium so as to create a venous

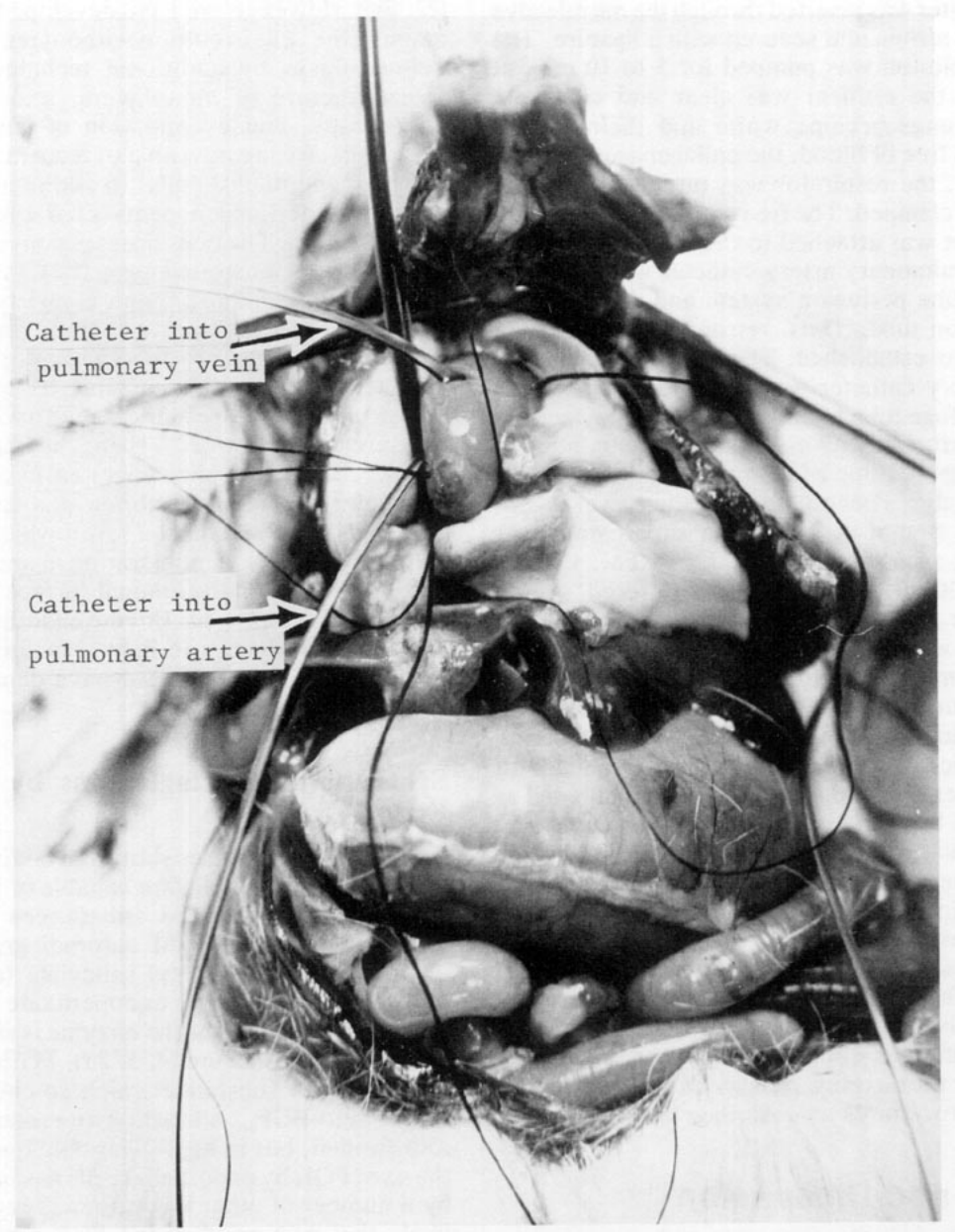


FIGURE 1. Perfusion system for rendering lungs free of blood and treating the microvasculature with collagenase to allow collection of endothelial cells. The perfusion system is described in the text. Small gauge catheters are inserted and tied into the pulmonary artery and pulmonary vein sinus. Saline is perfused in a forward direction (artery to vein) until the effluent is free of blood. Perfusion of enzyme is from the venous to the arterial side; a maneuver accomplished by connecting the drain cannula (in the pulmonary vein) to the enzyme stopcock and by disconnecting the cannula to the pulmonary artery from the saline stopcock. The free end of the arterial cannula is then directed to a collection tube.

sinus into which all pulmonary veins drained. The saline perfusion system was opened to the arterial catheter. The pulmonary artery was opened and the catheter was inserted and secured with a ligature. A hole was immediately cut in the right ventricle for drainage; a hole was also cut in the left ventricle and a drain catheter was inserted through the mitral valve and into the atrium and secured with a ligature. The perfusion solution was pumped for 5 to 10 min, at which time the effluent was clear and colorless. When the lungs became white and their venous effluent was free of blood, the collagenase stopcock was opened, the respirator was turned off and the trachea was clamped. The free end of the pulmonary vein catheter was attached to the collagenase stopcock. The pulmonary artery catheter was detached from the saline perfusion system and directed to a cell collection tube. Thus, retrograde perfusion of the lungs was established. The free end of the pulmonary artery catheter was directed into a sterile 15 ml centrifuge tube to collect the cell-enzyme suspension. Perfusion with collagenase was continued until the lungs became edematous and flow stopped spontaneously. The collection tubes were centrifuged for 10 min at 750g. The supernatant was removed by aspiration, and each pellet was resuspended in 10 ml of 0.01 Hepes buffered saline, pH 7.4. The tubes were centrifuged again and the supernatant was discarded. The pellets were resuspended in 1 ml of medium 199 using a Vortex mixer. Corning tissue culture flasks, 25 cm² were filled with 4 ml of medium 199 containing 20% fetal calf serum and antibiotics. Each culture flask was seeded with 1 ml of suspension and the flasks were incubated at 37°C in 100% humidity in a 5% CO₂ atmosphere. The number of cells seeded per flask is approx. 2.8×10^5 . However, the seeding density cannot be determined accurately since the cells are removed as clumps or sheets, not as a monodispersion and because blood cells are present (and beneficial) in the original isolate. Those flasks which contain more than 95% endothelial cells (as judged by phase contrast microscopy) are retained for culture and the lines are purified over the next few passages using differential adherence procedures as described above.

Results and Discussion

Identification of Endothelial Cells

Cells obtained by either method are readily identifiable as endothelial using morphological and functional criteria. When examined in the inverted microscope by phase contrast microscopy the cells grow as monolayers with a cobblestone appearance characteristic of endothelium (Figs. 2 and 3). When

examined in the electron microscope, they contain all of the cellular organelles expected of pulmonary artery endothelial cells *in situ* including Weibel-Palade bodies (Figs. 4 and 5). The cells are routinely examined by electron microscopy of thin sections of monolayers still attached to the culture flasks (22, 24). However, we have developed means of examining the cells in culture without removal from the culture flasks by additional techniques including freeze-fracture of monolayers, scanning electron microscopy, and examination of surface replicas (25). Thus, we are now able to recognize a variety of views of endothelial cells. In addition, our cultures possess functional aspects characteristic of endothelial cells. The cells possess converting enzyme activity as demonstrated using [¹²⁵I]Tyr⁸-bradykinin as substrate (22, 26, 27) and a variety of synthetic substrates (please see accompanying paper). In addition, the cells are reactive with antibodies to angiotensin converting enzyme as can be shown using immunofluorescence microscopy and immunocytochemistry (22). Using the latter technique, we were able to localize angiotensin converting enzyme on the plasma membrane and caveolae of endothelial cells in culture, a result which is in accord with our previous demonstration of angiotensin converting enzyme on the luminal surface of pulmonary endothelial cells *in situ*. Bovine endothelial cells also react with antibodies to human factor VIII and α_2 -macroglobulin, and this we have demonstrated by immunofluorescence (24).

Synthesis of Prostaglandins by Pulmonary Endothelial Cells

Results of our studies show that bovine pulmonary artery endothelial cells are capable of forming prostaglandins and related substances from 1-¹⁴C-arachidonate. Using EM autoradiography of cells incubated with ³H-acetyl salicylate (a specific inhibitor of prostaglandin endoperoxide synthase) we were able to show that the enzyme is situated on the endoplasmic reticulum (4, 5, 28). PGE₂ is the major metabolite. A substance which co-chromatographs with 6-keto-PGF_{1 α} , a breakdown product of PGI₂, is also formed, but in highly variable quantities. Synthesis of PGI₂ by endothelial cells has been described by a number of other laboratories recently (7). It is likely that part or all of the PGI₂ formed by lungs arises from endothelial cells. Synthesis of PGI₂ by pulmonary endothelial cells, along with their ability to degrade ADP (please see below), may account in large part for the capacity of endothelial cells to prevent or inhibit platelet aggregation. We were surprised to find that PGB₂ occurs as a major product. Conceivably, PGB₂ is formed from PGE₂ during ex-

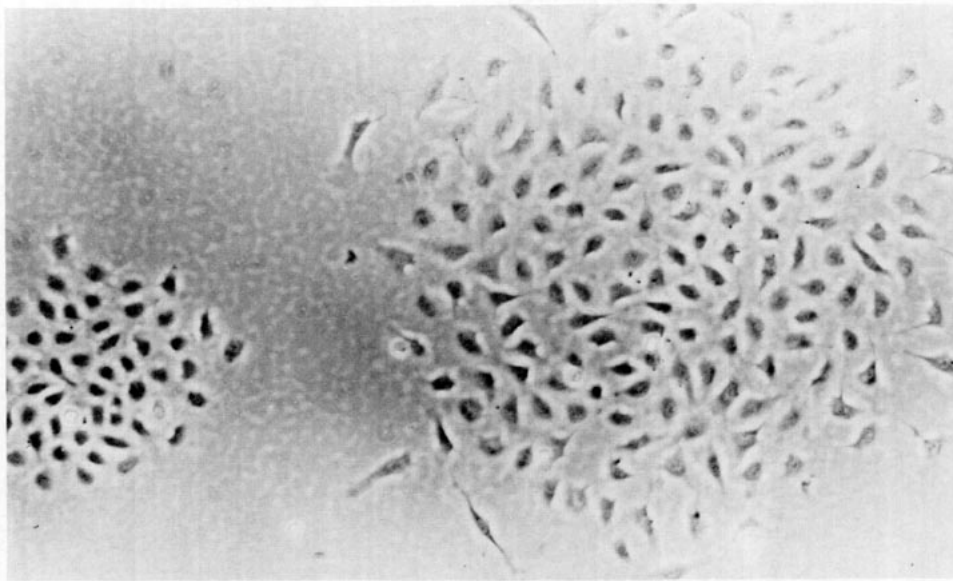


FIGURE 2. Phase contrast micrograph of cow pulmonary artery endothelial cells after 21 hr in culture. The cells form a cluster of ca. 100 cells per cluster. $\times 150$.

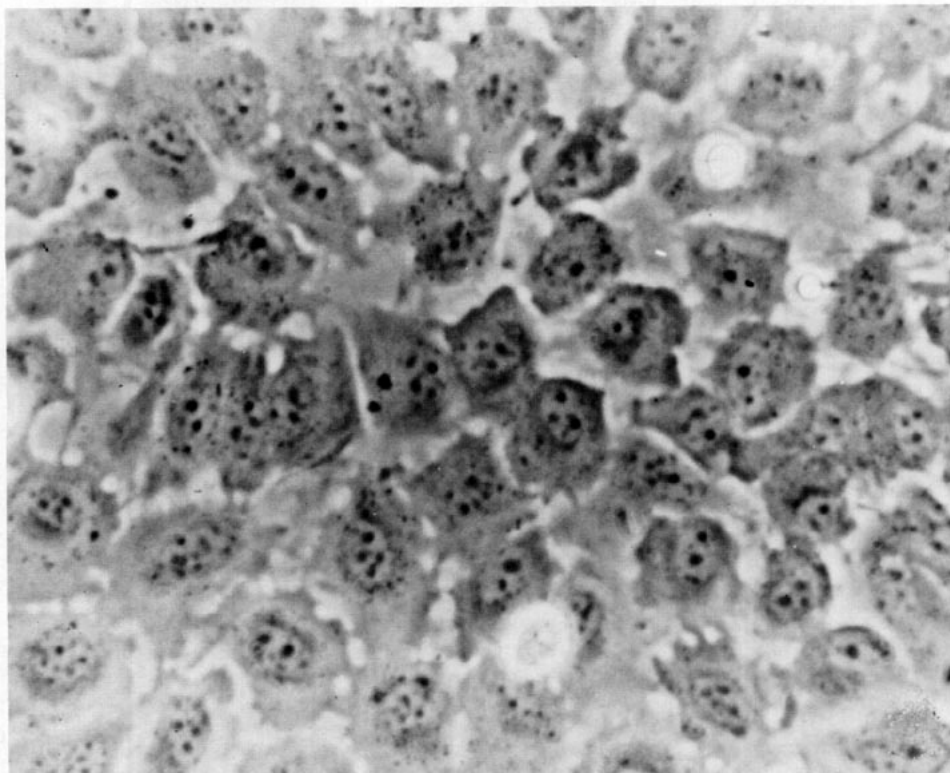


FIGURE 3. Confluent monolayer (ca. 4×10^4 cells/cm²) of cow pulmonary artery endothelial cells. Phase contrast. $\times 750$.



FIGURE 4. Electron micrographs of calf pulmonary artery endothelium sectioned transversely including a portion of the plastic culture flask on which the cells were growing (F). The boundary between the flask and the cell is delineated by a fine dense line indicated by an arrow (probably formed from components of FCS) (see also Fig. 5). (a) Overall appearance of a monolayer in transverse section, illustrating the contact inhibition between neighboring cells, $\times 1680$. Main body of the cell containing rough (*) and smooth ER. Golgi complexes (G) are frequently found around the nucleus (N). $\times 8800$. Thick portion of the cell in the region of the nucleus and the slender peripheral extensions. These extensions do not contain abundant endoplasmic reticulum but are characterized by numerous microtubules and microfilaments. Caveolae (arrowheads) occur on both the peripheral and central portions of the cells. $\times 5500$. This figure is reproduced with permission from Ryan et al. (24).

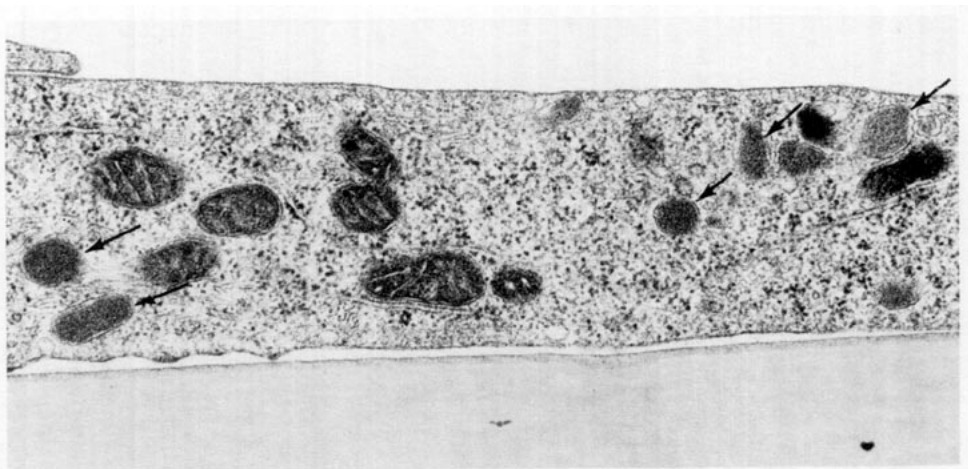


FIGURE 5. Thin section of endothelial cell showing Weibel-Palade bodies (arrows). $\times 32,000$.

traction. If so, the rate of PGE_2 synthesis approached $0.5 \text{ ng}/\mu\text{g}$ of cell protein per hour. The possibility should be considered that pulmonary artery endothelial cells have an unusually large capacity for synthesizing PGE_2 , a vasodilator thought to prevent or lessen hypoxic pulmonary vasoconstriction. Our results indicate that pulmonary endothelial cells can account for part of the efflux of prostaglandin-like substances from embolized or anaphylactic lungs. However, our results provide no information on the cellular origins of thromboxane A_2 , likely to be a primary mediator of anaphylaxis, or of substances such as 15-ketodehydro metabolites of PGE_2 and $\text{PGF}_{2\alpha}$, all compounds which may occur in lung venous effluent in much higher concentrations than those of PGE_2 and $\text{PGF}_{2\alpha}$. Previously, we have shown that endothelial cells in culture cannot degrade $\text{PGF}_{1\alpha}$ and E_1 , excellent substrates for 15-OH-dehydrogenase (3).

Endothelial Cells and the Degradation of ADP

Intact blood vessel walls are known to have antithrombotic properties, properties thought to be associated with the endothelial cell lining layer. As mentioned above, it has recently been shown that endothelial cells in culture can synthesize prostacyclin (PGI_2), a substance which can prevent platelet aggregation and which can also disaggregate platelet clumps. The lungs can also degrade the potent, platelet aggregating agent adenosine 5'-diphosphate (ADP) (29). Thus, in conjunction with our studies on prostaglandins and related substances we have examined pulmonary artery endothelial cells in culture for ADPase activity. Approx. 10^6 cells were incubated with ^{14}C - or ^3H -ADP (with or without carrier) at 37°C in 5 ml of culture medium. At

timed intervals, the incubation medium was examined by thin-layer chromatography. ADP at concentrations of $3 \times 10^{-7}\text{M}$ to $3 \times 10^{-5}\text{M}$ was found to have a half-life of approximately 3.5 min. The first product formed was 5'-AMP, which was itself degraded to yield adenosine and inosine. Adenosine did not accumulate in the medium but was taken up by the cells and incorporated into ADP and then into ATP. In a second series of experiments, endothelial cells were removed from their flasks with a rubber policeman and homogenized. Subcellular fractions were prepared by differential centrifugation. ADPase activity occurred most abundantly in the microsomal fraction, the fraction also enriched in terms of angiotensin-converting enzyme (ACE). ACE is known to occur as a component of the plasma membrane of endothelial cells (24). Thus, pulmonary endothelial cells can act to prevent intravascular thrombosis by degrading ADP. The ADPase activity is probably a component of the cell membrane.

Synthesis of Angiotensin-Converting Enzyme by Endothelial Cells in Culture

Recently we undertook a study to determine whether endothelial cells in culture synthesize angiotensin converting enzyme, or whether they merely provide highly specific receptors for the enzyme. Previously, we have shown that endothelial cells from cow, pig, rabbit and rat possess angiotensin converting enzyme, and we have shown that the enzyme occurs along the plasma membrane and associated caveolae (22, 27, 30). Further, we have shown that the angiotensin converting enzyme activity persists through more than 50 passages (3). Initially, we took these data to mean that endothelial cells in culture are capable of synthesizing angiotensin-converting enzyme. However, all of our

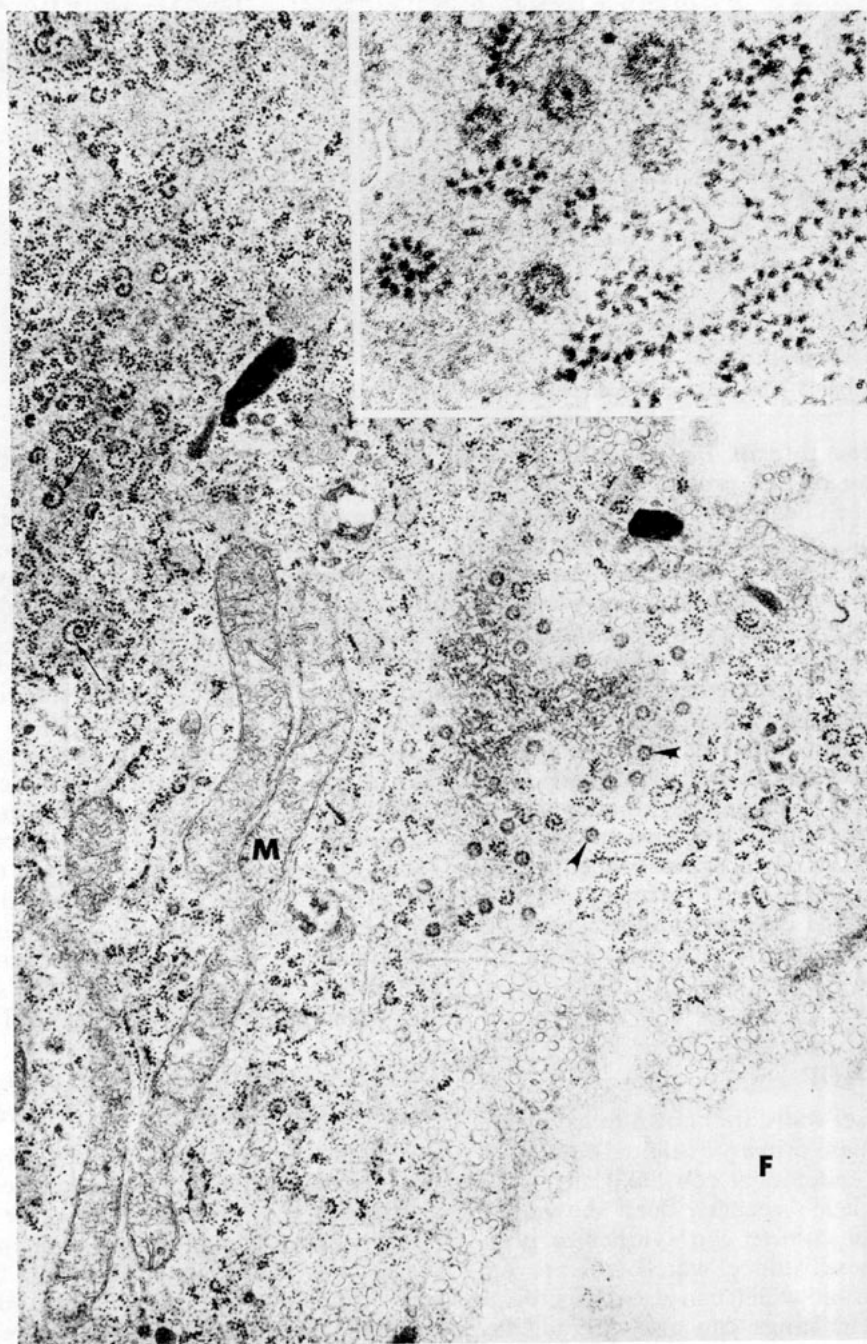


FIGURE 6. *En face* section of calf pulmonary endothelial cell spanning the cell from the culture flask (F) to the nucleus. The plane of section has grazed the nuclear envelope revealing nuclear pores (arrowheads) and also grazed the plasma membrane revealing abundant caveolae (*). In addition to elongated mitochondria (M), the cytoplasm contains large numbers of whorled polyribosomes (arrows) attached to the cisternae of the endoplasmic reticulum. The inset shows an enlargement of a portion of the field showing the spiral arrangement of ribosome complexes. $\times 22,000$; inset $\times 75,000$. Reproduced with permission from Ryan et al. (24).

previous studies used cells grown in medium containing fetal calf serum (FCS) at 20 to 30% v/v FCS. FCS is a rich source of angiotensin-converting enzyme; hence it seemed possible that endothelial cells provide receptors for, but do not synthesize, angiotensin-converting enzyme. To resolve this problem, we have obtained cultures of bovine pulmonary endothelial cells in FCS previously treated by heating at 56°C, a maneuver reported to inactivate angiotensin converting enzyme (31). The primary cultures were propagated through four passages, such that the average progeny in the fourth generation of each cell in the original isolate was 6,400. The cells were assayed by using [³H]benzoyl-Phe-Ala-Pro as substrate (32). The specificity of the assay was tested by using SQ 14,225 (10^{-5} M and 10^{-6} M), a specific inhibitor of angiotensin converting enzyme.

Enzyme activity per cell was remarkably constant, although there was a tendency for cell size to increase along with, in the fourth generation, an increase of enzyme activity per cell. Under the conditions of these experiments, there was a 12,428-fold increase in angiotensin converting enzyme activity. Thus, these results support the concept that bovine pulmonary artery endothelial cells in culture are capable of specific protein synthesis. Electron micrographs of grazing sections of the cells in culture suggest that the cells appear to have the subcellular machinery (extensive rough endoplasmic reticulum and polyribosomes [Fig. 6]) to synthesize a complex glycoprotein such as angiotensin converting enzyme (24).

Concluding Remarks

As described above, techniques for the isolation and culture of endothelial cells from bovine pulmonary artery have been established. These cultures have provided useful information on specific metabolic activities of pulmonary endothelium, and have the advantage that large quantities of cells can be obtained in original isolates. In addition, antigenic sites of bovine cells appear to cross-react with antibodies to human antisera. However, the techniques are not suitable for obtaining endothelial cells from small animals. Moreover, endothelial cells of pulmonary artery and those of the microvascular bed of the lungs *in situ* differ in terms of structure. In addition, the small caliber and large extent of surface area of the pulmonary microcirculation make it likely that processing of blood-borne substrates such as angiotensin, bradykinin, adenine nucleotides and amines is most likely to take place at the level of the smallest vessels. We have, therefore, developed a technique for obtaining endothelial cells from the lungs by vas-

cular perfusion with collagenase (500 units/ml) in HEPES buffered salt solution.

Although one can readily isolate and culture endothelial cells from pulmonary artery of, e.g., cow (22, 24, 27), and one can readily isolate and culture endothelial cells from human umbilical vein (3, 16), we believe that our newly developed techniques for obtaining endothelium from the lungs of small animals by vascular perfusion may have at least two effects that will accelerate the pace and broaden the scope of studies on specific metabolic functions of the lungs. Firstly, the vast preponderance of studies on the possible metabolic functions of the lungs have been performed using small animals; animals in which it is virtually impossible to obtain an adequate number of cells from mainstem pulmonary artery. Secondly, although we and others have shown that cells from main-stem pulmonary artery are capable of metabolizing bradykinin, angiotensin, ADP, adenosine, and biogenic amines, the general assumption is that the processing of such substances *in vivo* occurs most prominently at the level of the microcirculation. Our new techniques of cell isolation allow use of small animals and therefore allow a more direct comparison with previous functional studies in which small animals, such as rats and rabbits, have been used.

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